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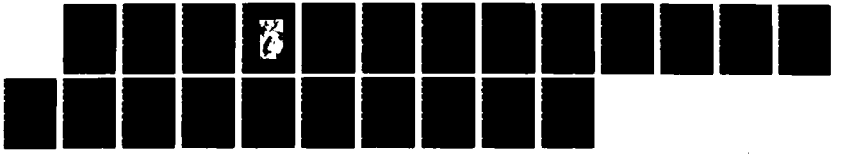

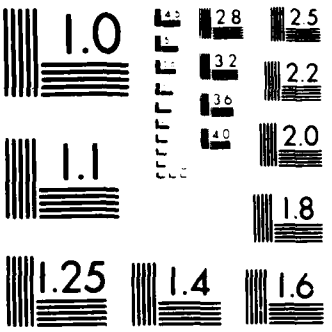

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Linear and non-linear dielectric properties of sickle cell anemia hemoglobin were investigated. Under normal conditions where sickle cell hemoglobin is distributed randomly in solution, their dielectric behavior is only slightly different from that of normal hemoglobin. When they form liquid crystalline gel, major portion of the hemoglobin S molecules become interlocked in gel lattice and dielectric constant decreases. However, the dielectric properties of free hemoglobin S which do not participate in gel formation, remain by and large unaltered. The presence of large gels imposes some constraints on the rotation of free hemoglobin. Hb S gels seem to be unstable mechanically and electrically. Relatively small mechanical shear is sufficient to cause degradation of the gel. Likewise, moderate electrical fields (10-20 V/cm) seem to cause underfined perturbation of gel lattices.

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FINAL REPORT ON CONTRACT N00014-K-0517

TITLE: INVESTIGATION OF NON-LINEAR DIELECTRIC BEHAVIOR OF SICKLE CELL HEMOGLOBIN

PRINCIPAL INVESTIGATOR: Shiro Takashima

INSTITUTION: University of Pennsylvania

Introduction

Sickle cell anemia is a hereditary disease which is found predominantly among black population in African continent and United States. In addition, this disease is found in Mediterranean countries. The pathogenesis of this disease is the propensity of abnormal erythrocytes (called SS erythrocyte) of the patient with sickle cell anemia to change its shape in microvasculature after oxygen is discharged from red cells (Dean et al. 1978). The ordinary discoidal red cells turn into a sickle-like shape. Upon sickling, SS erythrocytes increase rigidity and this in turn, increases the viscosity of whole blood.

Figure 1

The chain of events culminate in the occlusion of the capillaries and triggers the onset of painful episodes of sickle cell crisis. Repeated cycles of sickling and desickling causes damages in the membrane of erythrocytes and this results in marked decrease in the hematocrit in the patient with sickle cell anemia (Ortiz, et al. 1986, Lessin et al, 1978).

It is now known that the ultimate cause of sickling of SS-erythrocytes is nested in the abnormal polymerization characteristics of hemoglobin S (Hb S). Under normal conditions, i.e., in the presence of oxygen, the function and physical state of Hb S are almost identical to those of normal hemoglobin (Hb A). However, in the absence of oxygen, Hb S molecules become deoxygenated and form fibrous tactoids (this is called gel) (Josephs, et al. 1976, Dykes, et al., 1978).

Tactoids or gels are not crystals in the strict sense of the word. Although X-ray analyses revealed that the internal structure is highly ordered and by no means amorphous, never-the-less, tactoids have clearly distinguishable structure and properties from those of real Hb S crystals (Wishner et al. 1975). Tactoid is said to be a liquid crystal and as such its mechanical rigidity is very low. Inspite of this, the intracellular formation of gels is sufficient to deform the membrane of red cells. The ease with which erythrocyte deforms is due to the extremely high elasticity of its membrane (Evans, et al. 1979). Thus even a small force is sufficient to cause considerable deformation of red blood cells.

Figure 1. Scanning electron micrograph of normal shaped and sickled cells. Taken from Sickle Cell Fundamentals, The National Sickle Cell Disease Program, National Heart, Lung and Blood Institute, National Institute of Health, 1975.

It is now clear that the pathogenesis of sickle cell anemia stems from the abnormality of hemoglobin carried by the patient with sickle cell anemia. It is well known that the difference in the chemical structure between Hb A and Hb S is the replacement of two glutamic acids in Hb A with two valines in Hb S (Ingram, 1956). Since valine has a non-polar side chain while glutamic acid has a negatively charged side group, the replacement results in the loss of two charges in Hb S. The reduction of electrophoretic mobility of Hb S compared to Hb A is well known (Itano, 1951). In addition, the net dipole moment of Hb S is presumed to be smaller than that of Hb A. This is the first question we addressed at the outset of this project.

Secondly, formation of the tactoids causes the interlocking of individual Hb molecules in lattices. The contacts between neighboring molecules are weak hydrophobic linkages rather than covalent bonds. The energy of these weak bonds is quite small and perhaps less than 5-6 kcal/mol. However, each Hb S molecule is linked to neighbors by several contact points as shown by Fig. 2. Therefore, weak hydrophobic linkages, when summed, amounts to a considerable constraint of molecular motions. Under this condition, it is reasonable to assume that the freedom of translational and/or rotational diffusions of Hb S will be reduced markedly upon tactoid formation.

Figure 2

The reduced rotational freedom can be detected by various techniques such as spin label ESR experiment (Yamaoka et al., 1974) and also by the measurement of dielectric constant (Delalic et al, 1983) if the molecule is polar. Hemoglobin molecule is known to have a large dipole moment (480 Debye Units, Oncley 1943) and this manifests itself as an enhanced dielectric constant of solvent. When the rotation of dipoles is reduced by physical constraints such as freezing or crystallization, the dielectric constant will decrease markedly. This is a well known phenomenon for organic dipolar compounds. Thus, we gain considerable information and insights as to the dynamics of Hb S molecules in gel by measuring the dielectric constant of Hb S before and after the formation of tactpods. This is the second question we addressed in our proposal. In the following, I will describe the experimental procedures before the results of proposed experiments are presented.

Experimental Procedures

Apparatus ... Dielectric measurements were performed using two systems. The one is manually operated RF bridge (Wayne Kerr, Model B601) which covers a frequency range between 10KHz and 5 MHz. The oscillator used was a Hewlett-Parkard 606 RF generator and null detector was Rohde-Schwarz microvoltmeter type USVH. Although the Wayne Kerr bridge used was manually operated, its characteristics are well delineated and gave exceedingly reliable results in the frequency range of interest. Hemoglobin solution usually undergoes an anomalous dispersion between 100 KHz and 10 MHz. The dynamic range of the bridge ends around 5 MHz. This frequency limitation was not a serious problem because the dielectric constant of hemoglobin solution is very close to that of water at this frequency.

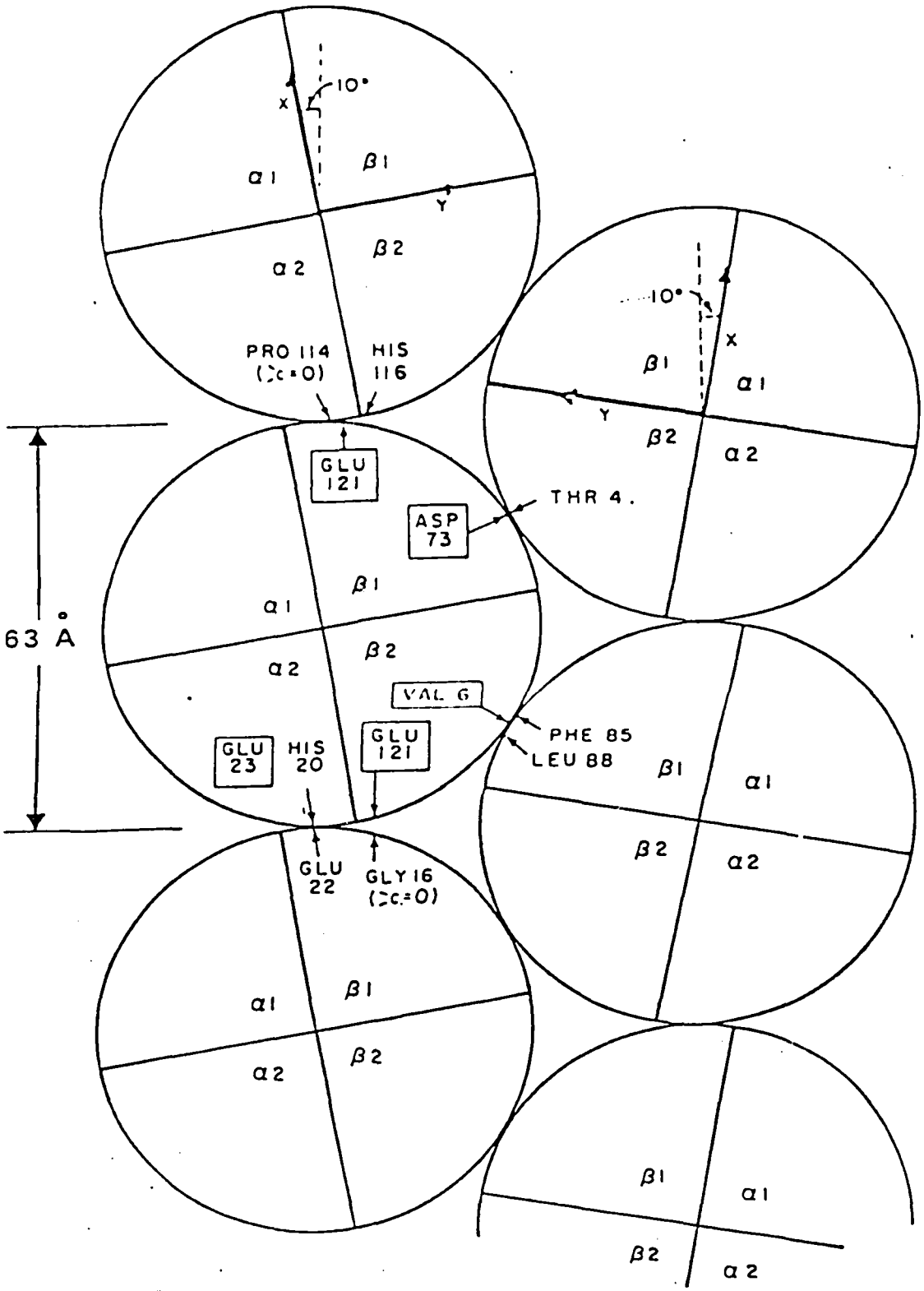

Figure 2. Intermolecular contacts between neighboring strands of HbS constructed from x-ray crystallographic data of Wishner et al (1975).

The second instrument used for this project is Hewlett Packard Impedance Analyzer model L4192A. This instrument is fully automated and is operated by an on-line HP 9816 desk top microcomputer. This instrument is fast and reasonably reliable. However, its internal configuration is not well understood which apparently contains some frequency dependent components. Therefore, great caution must be exercised not to exceed the capability of this system. Although this instrument has a nominal frequency range between 5Hz and 13 MHz, its actual usable range was found to be only between 10KHz and 10 MHz for aqueous protein solutions. Inspite of this limitation, this analyzer was quite useful for the measurement with hemoglobin solution and gel as well.

The dielectric cell used for the project is illustrated in Fig. 3. Electrodes are platinum rod which was coated with Pt-black to reduce electrode polarization effect. The cell was made air-tight so that we can replace ambient air with pure nitrogen.

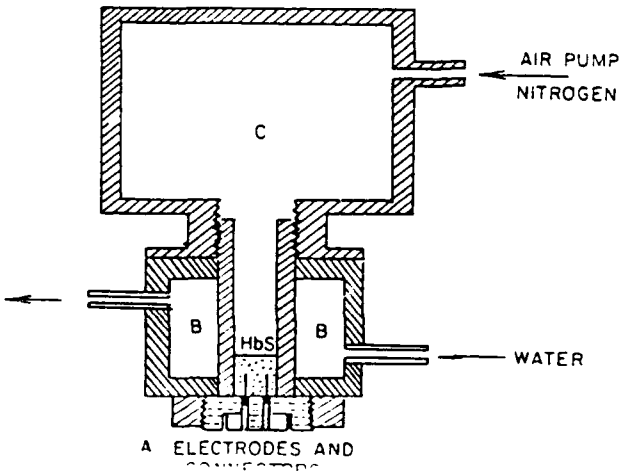

Figure 3. The electrode assembly used for the measurement of dielectric constant of Hb S solution and gel. A. electrodes and sample holder, B. Water jacket and C. Nitrogen compartment.

As stated before, Hb S forms tactoids only in the absence of oxygen, i.e. only deoxy-hemoglobin S can form fibrous polymers. Therefore, the measurement must be performed using an air-tight cell in nitrogen atmosphere. In addition, gel formation occurs at relatively high temperatures between 30-40^{o}C. The dielectric cell is surrounded by a water jacket for temperature regulation.

Viscosity Measurement ... Viscosity measurement has been used to follow gelation kinetics of Hb S (Harris et al, 1975). We used a rotating type Brookfield viscometer with sparging option so that sample compartment can be filled with nitrogen. Temperature is regulated by circulating thermostated water.

Sample .... Hb S was purified from SS blood or more often from AS blood. SS blood is very difficult to obtain in Philadelphia area. Therefore, we resorted to the people who are often called carrier. The blood of these people is 50% normal hemoglobin and 50% SS blood. Separation of S hemoglobin from A hemoglobin was implemented using DEAE Cephadex column using a fraction collector. SS blood samples were the donation from the Children's Hospital of Philadelphia and all of the biochemical operations were performed in the Hematology Division of the same hospital. The final purified sample was dialyzed against water to reduce conductivity and concentrated to 25-30%. It was found

that the sample after purification gelled almost instantly when deoxygenated (see Fig. 4).

Results

The gelation of Hb S has been investigated using a variety of experimental techniques. As mentioned earlier, gelation process can be followed by viscosity measurement and light scattering method (Briehl, 1987, Hofrichter, 1986). These techniques detect the appearance and growth of long polymers, whereas other methods such as dielectric technique follows the disappearance of free Hb S molecules. Both viscosity and light scattering are proportional to M^n (M is molecular weight and n is a numerical factor), the increase in molecular weight can be detected by these techniques. In addition, proton NMR (Eaton, 1976) has been used to study the kinetics of Hb S gelation. It has been known that gelation of Hb S reduces the rotational freedom of wate molecules in the vicinity of hemoglobin. Thus, the line broadening of proton NMR resonance signals can be used as the measure of the progress of gelation process. In the present project, two experimental methods are used, the one viscosity measurement and the other dielectric constant measurement.

Viscosity Measurement

The polymerization characteristics of Hb S was studied by viscosity measurement as a supplement to dielectric constant measurement. The results illustrated in Fig. 4 show that the viscosity of Hb S solution is of the order of 8-10 centipoises before gelation. This value is quite high for globular proteins such as hemoglobin. This is due to the fact that Hb S does not form gels unless its concentration is above a critical concentration, i.e., about 25-30%. Thus measurements have to be performed using a very high concentration of the sample. When nitrogen is introduced in the sample chamber, the viscosity begins to rise after some delay to an enormous value of more than 100 centipoises, an increase of more than 10 fold. As indicated by an arrow when oxygen is reintroduced, the viscosity decreases to the normal value without a delay this time. Curve 1 was obtained with a velocity gradient of 22.5/sec. However, the increase in velocity gradient results in a marked increase in delay time and a decrease in the peak viscosity as shown by curves 2 and 3.

Figure 4

This observation indicates that the viscosity of Hb S gel is highly non-Newtonian. In other words, the mechanical flow gradient which is used for viscosity measurement can cause partial degradation of the sample. Therefore, the real viscosity of gel must be determined at the limit of zero shear rate. Fig. 5 shows the peak viscosity of gels against velocity gradient. The intercept between this plot and the ordinate (log n) is found to be 200 cps. Thus viscosity measurement provides an unequivocal evidence of gel formation and at the same time, indicate the mechanical fragility of HB S gels. It has been postulated that Hb S gels are similar to liquid crystals. Our results confirm this postulate.

Figure 5

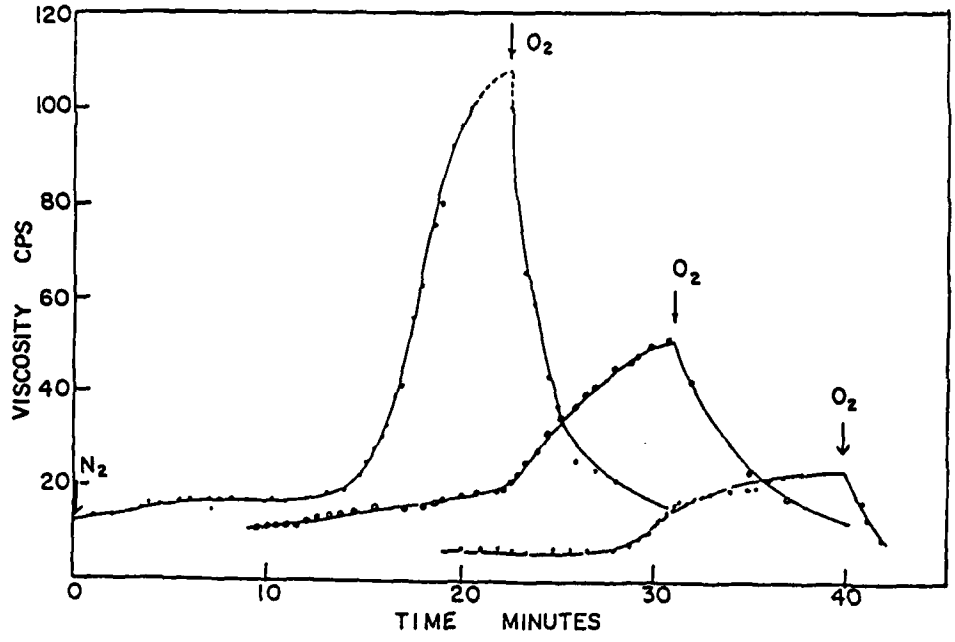

FIGURE 4

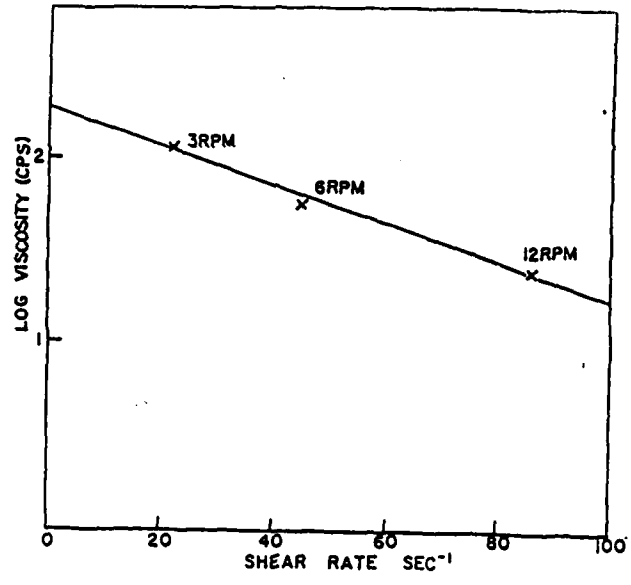

FIGURE 5

Figure 4 The viscosity change of Hb S upon gelation. Curve 1 was obtained with 3 PPM, curves 2 and 3 were obtained with 6 RPM and 12 RPM respectively.

Figure 5 Dependence of the viscosity of Hb S gel on shear gradients. Note that the ordinate is in logarithmic scale. The intercept between the plot and ordinate is about 200 CPS.

Dielectric Constant of Hb S and Hb A Solutions

The purpose of dielectric measurement with abnormal hemoglobin S is (1) to compare its dipole moment with the moment of normal hemoglobin and (2) investigate its rotational characteristics of Hb S before and after gelation.

The dielectric properties of normal hemoglobin has been investigated by many people and approximate values of the dielectric increment, dipole moment and relaxation time have been known. Originally, Oncley (1943) determined the dielectric increment of horse hemoglobin and found a value of 0.44/g/lit. This value gave a dipole moment of 480 D.U. assuming a molecular weight of 68,000 daltons. However, for unknown reasons, this value has not been confirmed by later investigations. The dielectric increment determined by other investigators are consistently lower than the one found by Oncley. Even now, there is no concensus about the value of dielectric increment of hemoglobin molecule. One of the reasons for this inconsistency is that the dielectric properties of hemoglobin depends on the method of purification, type of blood and the nature of ligands. Under these circumstances, it is necessary to reinvestigate the dielectric increment and relaxation time of normal hemoglobin each time as the control. Since the preparation of Hb S starts with mixed AS blood, we obtain Hb A as a byproduct. Thus, we are able to determine the dielectric properties of both Hb A and Hb S simultaneously. For this study, we must avoid the condition which might induce the gelation of Hb S (below critical temperature and concentration) so that the dielectric properties of normal and abnormal hemoglobins can be compared unequivocally.

Table I shows the dielectric increments and dipole moment of Hb A as well as Hb S. This table clearly demonstrates that the dielectric increment and dipole moment of Hb S are smaller than those of Hb A. As mentioned earlier, the difference between Hb A and Hb S is the loss of two negative charges due to the replacement of glutamic acids with valine residues.

Table I

The dielectric increment and dipole moment of normal hemoglobin (Hb A) and sickle cell anemia hemoglobin (Hb S).

	Dielectric Increment	Dipole Moment
Hb A	0.1803/g/lit	310 D.U.
Hb S	0.0746/g/lit	199 D.U.

It is well known that the dipole moment of protein molecules is, by and large, determined by the distribution of surface charges rather than the vector sum of bond moments. Therefore, the change in surface charges results in the alteration of the net dipole moment of entire molecule. The loss of only two surface charges may not be sufficient to account for the differences between A and S hemoglobins owing to the charge loss. Whether this difference in dipole moments has any relevance on the polymerization characteristics of Hb S is still unknown.

Dielectric Constant of Hb S Gel

Although the polymerization characteristics of Hb S and internal structure of the gel is already known, the dynamics of individual Hb molecules in gel lattices are not well understood. In order to investigate the rotary motions of Hb S in gel, Yamaoka et al (1974) used a spin label ESR technique. A similar study was performed by Asakura independently (Unpublished data). Spin label compounds are attached to hemoglobin molecules tightly and they do not rotate independently of the motion of the entire protein molecule. Therefore, the ESR signals of spin labels reflect the rotary motion of protein molecules rather than the rotation of spin compounds. Since gelation interlocks Hb S molecules, it was expected to find ESR signals drastically altered upon gelation. However, none of the studies conducted by these investigators found noticeable changes in ESR signals. This means that either the majority of Hb S are left free or the rotation of Hb S molecules is unhindered inspite of interlocking in gel lattices. There is no satisfactory explanation for this puzzling observation. In any event, this finding indicates enormous complexity of the gelation of Hb S.

Dielectric constant of polar molecule is due to the rotation of dipoles. Therefore, the origin of dielectric relaxation and ESR are due to a similar molecular dynamics. Therefore, we have reasons to believe that the results of dielectric measurement may be similar to those of spin label experiments. Fig. 6 illustrates the transition of the dielectric relaxation of Hb S solution to that of gel. For example, the diagram A shows the relaxation of Hb S solution immediately after deoxygenation. The diagram B illustrates the relaxation of the same sample a few hours later. Diagram C after several hours when it was presumed that gelation was complete.

Figure 6

Clearly, these figures show that the dielectric increment of Hb S decreases upon gelation. Obviously, this decrease is due to the interlocking of Hb S molecules in gel leading to drastic reduction of the rotational freedom of Hb S molecules. The figures also show that Hb S gel still exhibits a small but well defined dielectric relaxation even after a long waiting (24 hrs.). The most reasonable interpretation of this observation is that the residual dielectric increment is due to free molecules which do not participate in gel formation. Fig. 7 shows the decrease in the dielectric increment as the function of time. According to this figure, about a half of Hb S molecules are interlocked in gels and remaining molecules are free to rotate in solution. The results presented in Figs. 6 and 7 clearly demonstrate that the rotational motion of Hb S is reduced substantially when they are interlocked in gel. This observation is not in agreement with those of spin label experiments.

Figure 7

The measurements of dielectric constant were performed using small input AC signals. In view of the mechanical instability of gel structure, it was presumed that even moderate intensities of

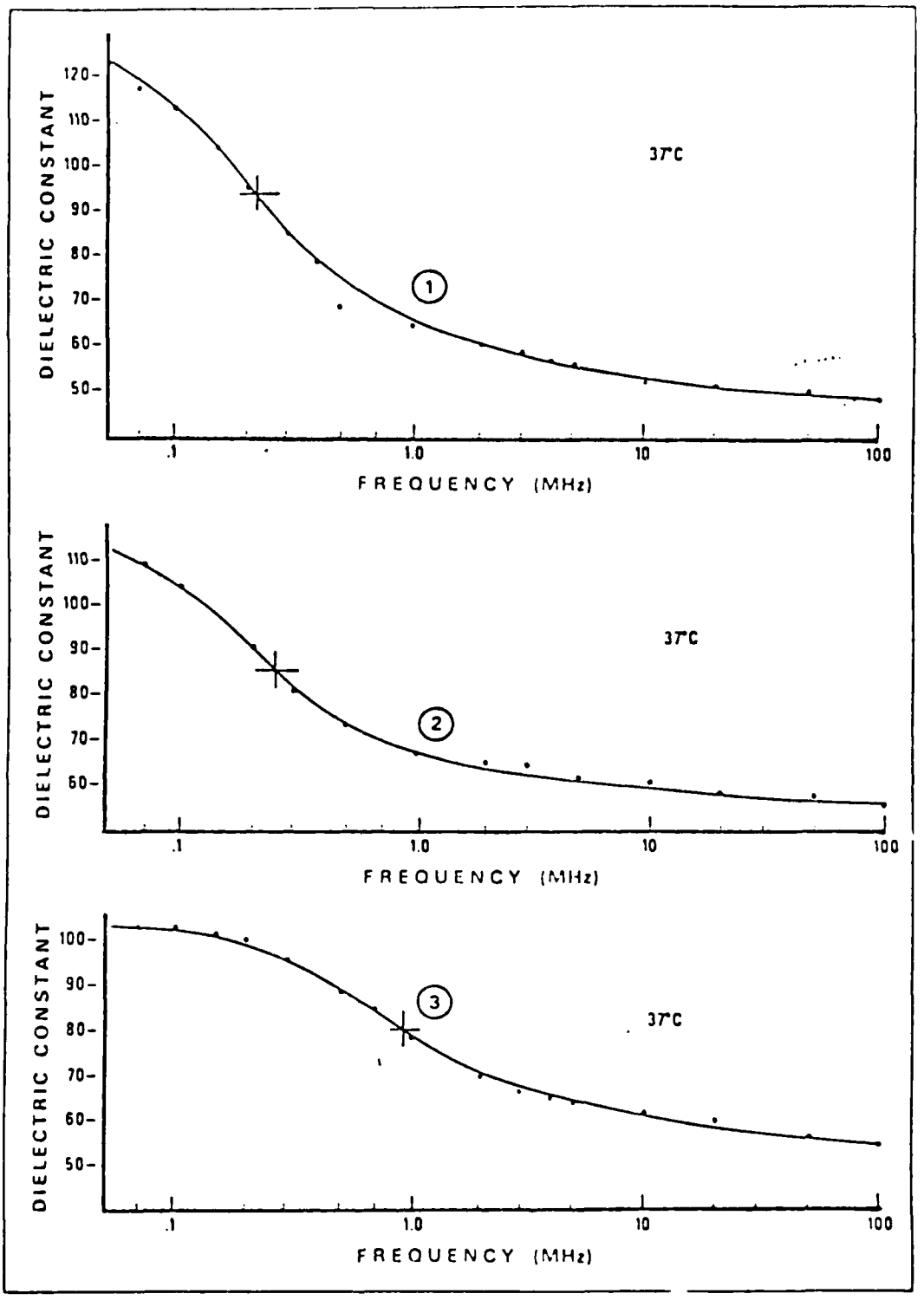

Figure 6 Dielectric dispersion curves of Hb S. Curve 1, Immediately after deoxygenation. Curve 2, 1 hour after deoxygenation. Curve 3, was obtained 4 hours after deoxygenation. When gelation is completed, the low frequency limiting value of reduces to about 90. Viscosity measurement indicates that gelation is complete within 20-30 minutes. Rotational motions last much longer after gelation. Temp. 37°C. Conc. of Hb S is 29 g/dl.

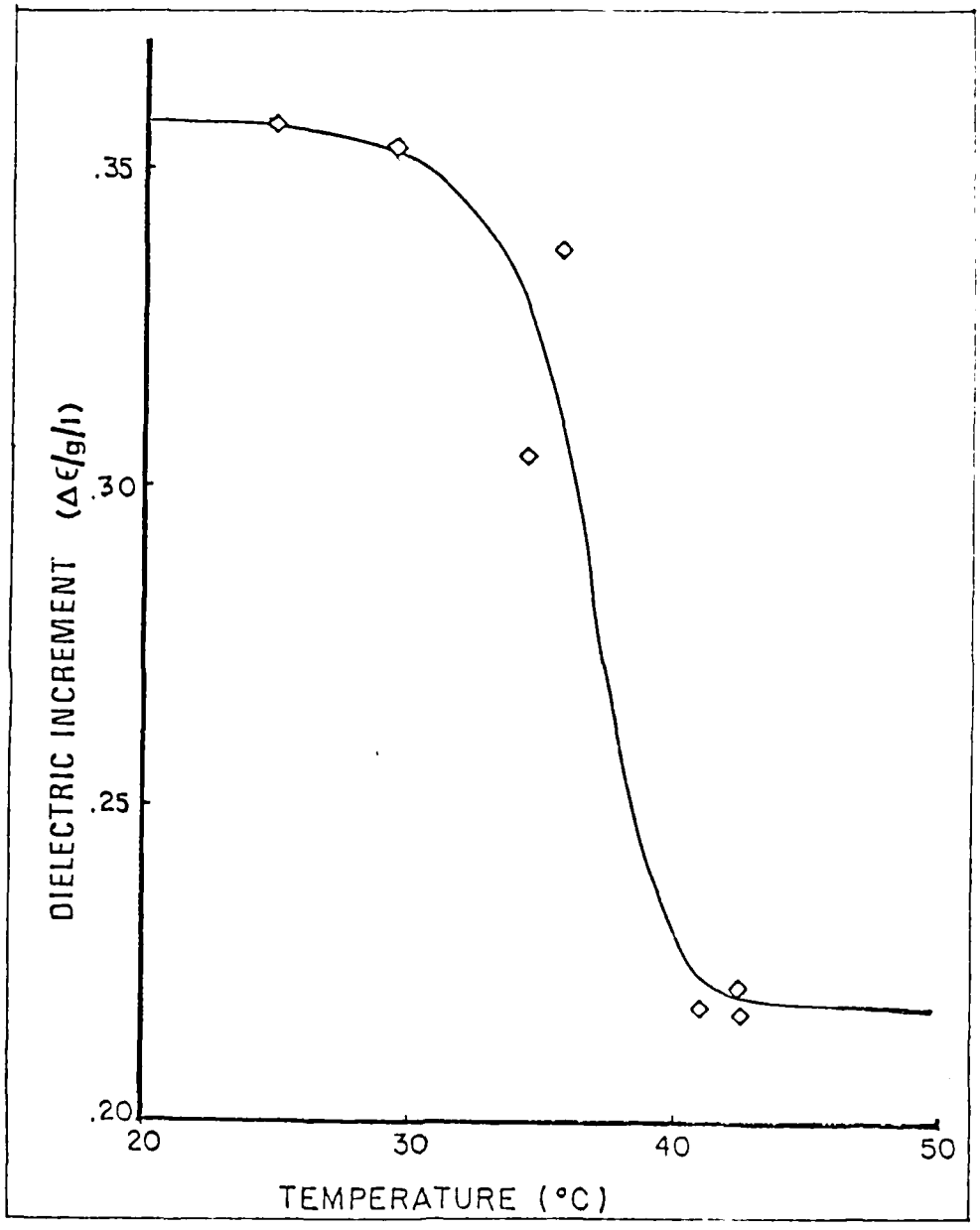

Figure 7 The change in dielectric increment of Hb S upon gelation. The data were taken with sufficient waiting time at each temperature. The decrease in dielectric increment is 38% of total increment.

evidence that Hb S gel is not only mechanically but also electrically unstable. Fig. 8 shows the results of a series of measurements in which the intensities of input AC signal were systematically varied. As illustrated by these figures, the frequency profile of the dielectric constant of Hb S gels gradually returns to that of solution with the increase in input voltages.

Figure 8

At a field intensity of 20 V/cm, the dielectric relaxation of Hb S is almost identical to that of Hb S solution. Clearly, the transition takes place at relative small input field intensities. At this juncture, I would like to speculate on the non-linear or field dependent dielectric properties of polar molecules.

Most of the dielectric measurements are performed using relatively weak input signals so that the measured dielectric constant is independent of the intensity of applied fields (Debye, 1929, Onsager, 1934). The origin of non-linearity is two fold. The one is the use of high intensity fields as an input and the other is instability of the molecule under investigation. The non-linearity due to high fields depends on the magnitude of dipole moment as shown by the following equation.

EQUATION (1) $$\langle m \rangle = \mu \left[\frac{\mu E}{3kT} - \frac{1}{45}\left(\frac{\mu E}{kT}\right)^3 + \frac{2}{945}\left(\frac{\mu E}{kT}\right)^5 - \cdots \right]$$

where <m> is mean moment of a group of dipoles, μ is the dipole moment of individual molecule, E is field intensity. (Polarizability is defined by the following equation, α = <m>/E). If the ratio E/kT is negligible, we can safely ignore the higher order terms and polarizability is independent of input voltage. However, when E increases, the magnitude of second order terms becomes increasingly large and we can no longer neglect it. Thus polarizability becomes voltage dependent i.e., non-linear. For proteins the dipole moment is typically 500 D.U. Therefore, the onset of non-linearity is of the order of 50,000 to 100,000 v/cm. For DNA which has a dipole moment of 10^5 to 10^6 D.U. (Takashima, 1967 and Wada et al, 1976) the onset of non-linearity is much lower and is of the order of 100-200 v/cm. The field intensity which is used in our experiment is only 10-20 v/cm, the dipole moment of Hb S tactoids must be at least 10 times larger than that of DNA if dielectric saturation effect is the only cause of non-linearity.

The second cause of non-linearity is the instability of molecules. If the structure of molecules is very unstable and applied fields can alter the internal configuration of the molecule and if this results in the change of dipole moment, measured dielectric constant will be dependent on input voltages. In view of the mechanical instability of Hb S, we have reasons to believe that a field of 10-20 v/cm may be sufficient to degrade the structure of Hb S gels. The non-linearity due to chemical relaxation was treated extensively by Bergmann et al. (1963) and Parsoons, et al (1978). In the original theory, they dealt with a field induced chemical reactions which require large electric fields. However, later Schwarz (1968) found that if molecules are in a critical state, even weak fields are sufficient to cause chemical relaxation which manifests itself as a dielectric

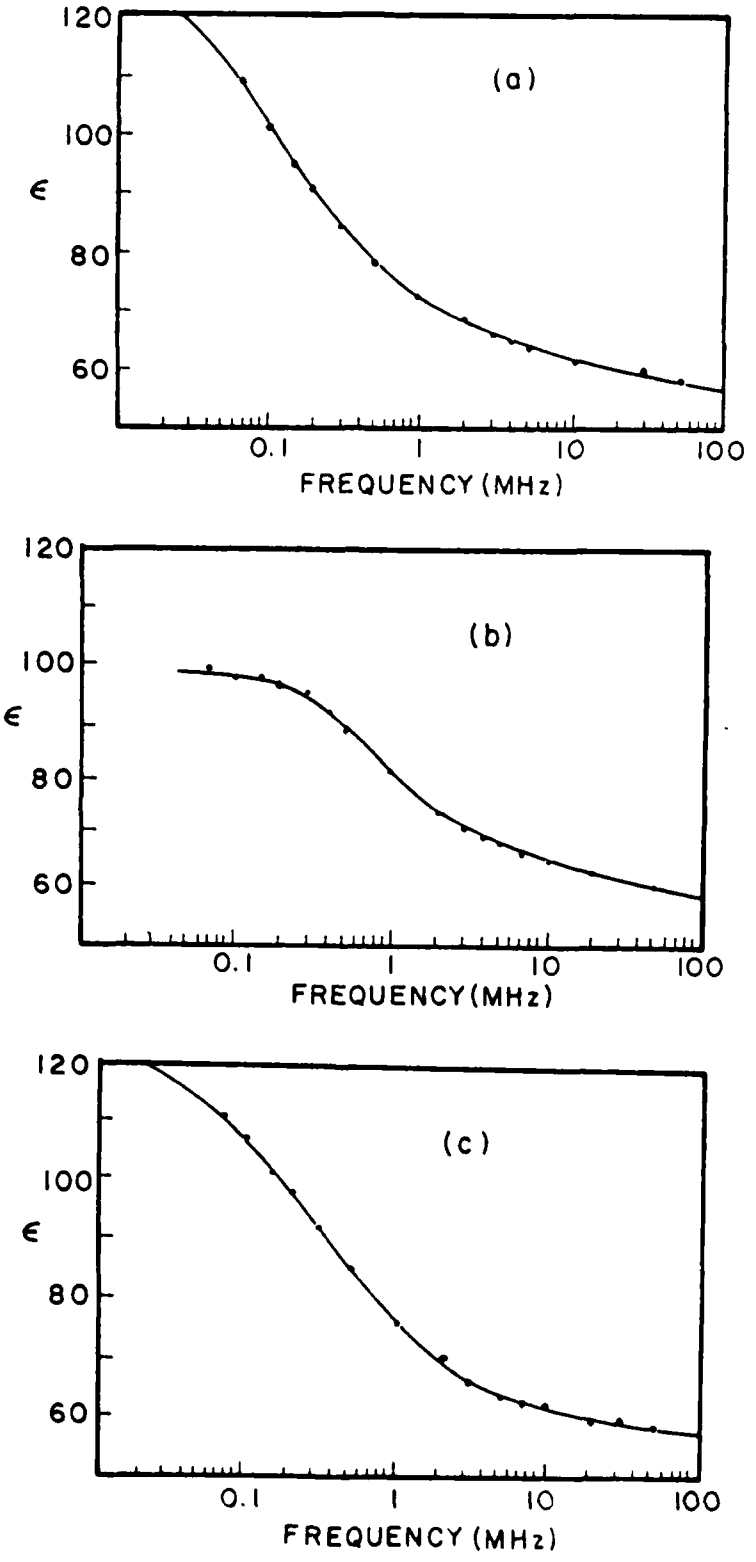

Figure 8 The dielectric dispersion of Hb S. (a) Oxv Hb S in solution. (b) Hb S gel measured with 500 mV/cm and (c) Hb S measured with 10V/cm. Note the similarity between (a) and (c).

relaxation if certain conditions are satisfied. Whether the non-linear dielectric behavior of Hb S gel is due to electrically induced chemical relaxation or due to simple saturation effect remains to be seen.

Thermodynamic Properties

Previous discussions indicate that there are considerable amounts of free hemoglobin molecules in Hb S gel and that the rotational characteristics of these molecules are not altered by gel formation. However, careful examination of these diagrams indicates some subtle differences between the rotational behaviors of Hb S before and after gel formation. In order to make more detailed comparision, it was decided to determine rotational energy of the Hb S dipole using Eyring's rate theory applied to dielectric relaxation. According to Eyring's theory (1941), the orientation of a dipole from one equilibrium position to another upon application of a field must overcome a potential barrier as illustrated by the inset of Fig. 9. The height of potential barrier is by definition the enthalpy of activation. This quantity can be determined by the slope of the plot of ln (t.T) vs 1/t as shown by the following equation.

$$\Delta H = R \frac{\partial Ln(T \times \tau)}{\partial(1/T)} \qquad (2)$$

where R and T have usual meaning, τ is relaxation time. According to this equation, the plot of lnτxT vs 1/T is linear unless there are configuration changes. We measured the relxation time of Hb S sample at temperatures between 10°C and 45°C and determined the slope of the plot of ln xT vs 1/T. Between 10 and 25°C, as shown in Fig. 9, the plot for non-gel oxy-Hb S is linear for the entire temperature range giving

Figure 9

rise to an activation enthalpy of 2-3 kCal/mol. This is a typical value for the protein solution of globular shape. For (free) deoxy Hb S in the presence of gels exhibits a steeper slope at high temperatures giving rise to an activation enthalpy of 5-6 kcal/mol. This is the activation enthalpy for Hb S dipoles to orient in the presence of a large amount of gels. This activation enthalpy indicates the energy which is requried for dipoles to rotatate and is proportional to the number of bonds broken by the rotation. The fact that the rotation of Hb S requires 6Kcal/mol, a value three times more than before gel formation, indicates that the interactions between polymeric gels and free Hb S molecules are restricting the orientational freedom of the latter. This conclusion is somewhat different from the one by Hoffrichter (1986) that the light scattering power of free Hb S in gel is nearly identical to that before gelation.

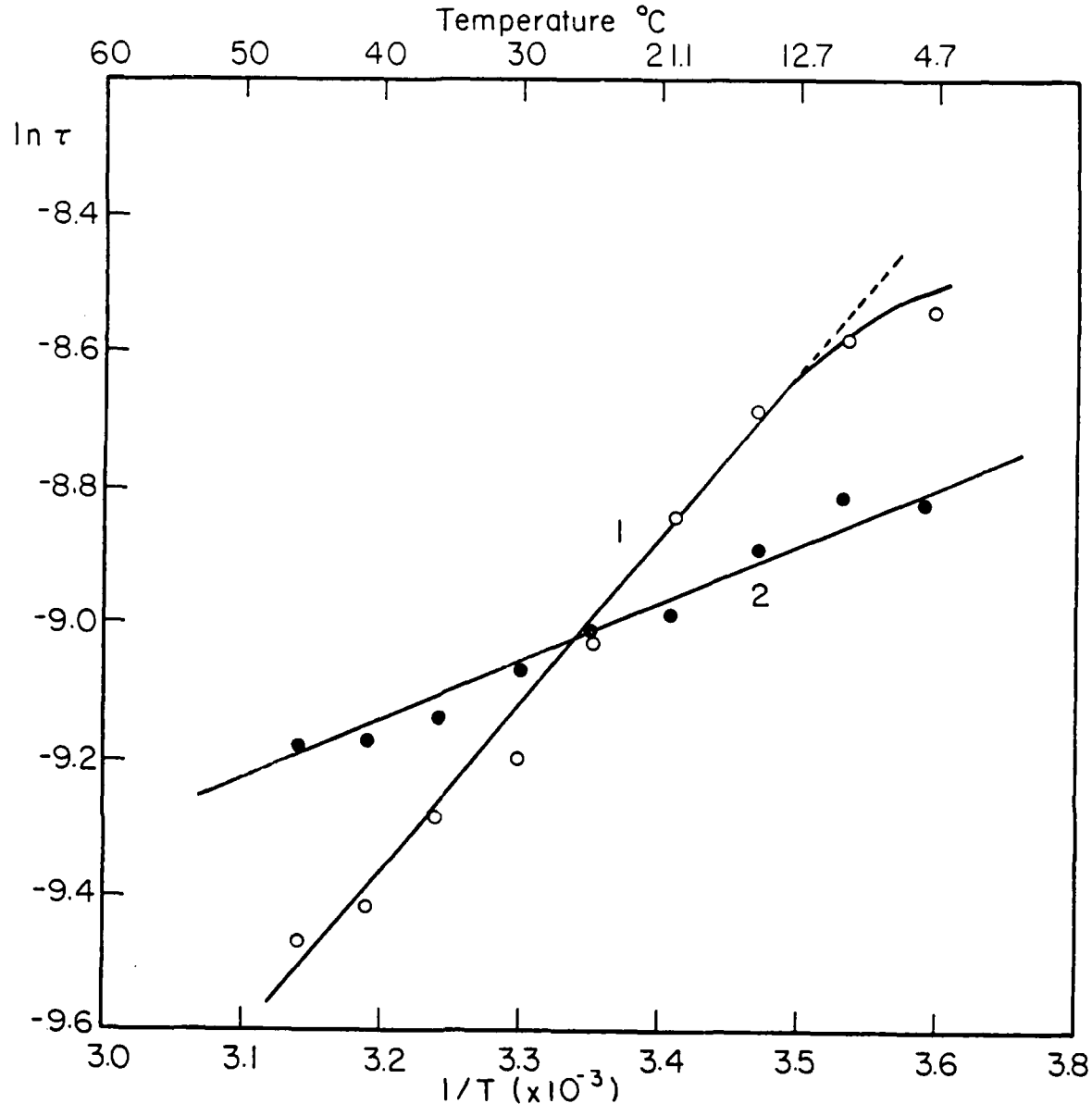

Figure 9 Eyring plot for Hb S in solution (curve 2) and in gel (curve 1). Lower abscissa shows 1/T and upper abscissa indicates temperature T.

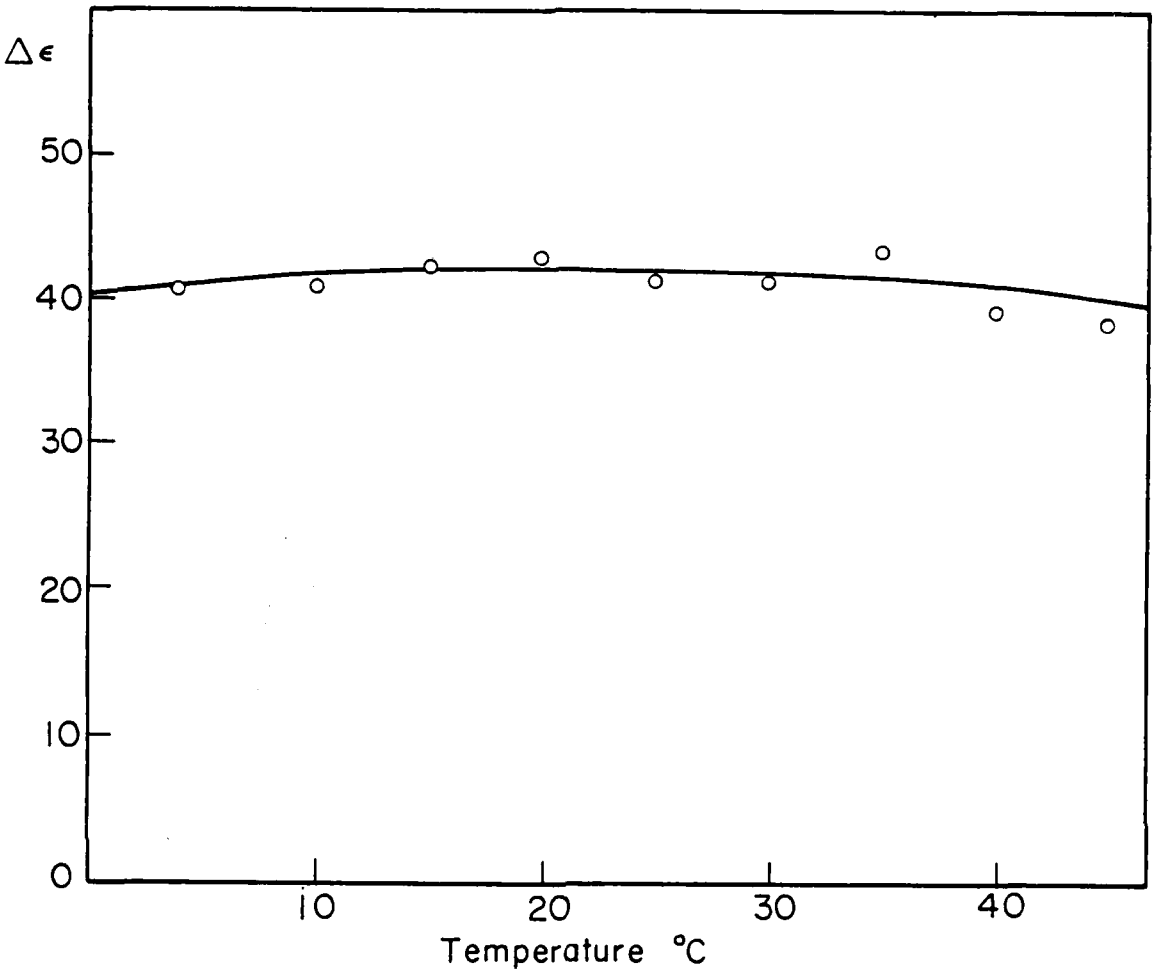

Figure 10 Dielectric increments of Hb S at various temperature. Note that gel transition has no effect on the dielectric increment.

Most of the dielectric measurements were performed using the samples prepared by ourselves using the facility at the Children's Hospital. However, we carried out additional measurements using a sample purchased from Wayne State University. Viscosity measurements confirmed that the gelation characteristics are almost the same as that of our own sample.

However, the dielectric behavior of this sample is somewhat different. The plot of dielectric increment against temperature is shown in Fig. 10. Clearly, gelation of the Hb S does not cause even a small decrease in dielectric increment and the increment of gel is virtually the same as that of solution. This puzzling results are, however, in agreement with the equally puzzling results of spin label experiments by Yamaoka and Waterman. It is generally agreed that the concentration of free Hb S molecules in gelled sample at the concentration of 35-37% is no more than 40% of the total solute. Thus our observation along with those of spin lable experiments indicate that not only free Hb S molecules but also those interlocked in gel lattices have a high degree of rotational freedom.

We were unable to reach a definitive conclusion as to the dielectric properties of Hb S because of these conflicting experimental data. Although polymerization characteristics of these samples are almost the same, small differences in their purities may have caused different dielectric behaviors of Hb S samples. Purity analysis of these sample may reveal some clues to this puzzling results.

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